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Innate immunity in *Drosophila*: Pathogens and pathways

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Abstract

Following in the footsteps of traditional developmental genetics, research over the last 15 years has shown that innate immunity against bacteria and fungi is governed largely by two NF- κ B signal transduction pathways, Toll and IMD. Antiviral immunity appears to stem from RNA interference, whereas resistance against parasitoids is conferred by Toll signaling. The identification of these post-transcriptional regulatory mechanisms and the annotation of most *Drosophila* immunity genes have derived from functional genomic studies using “model” pathogens, intact animals and cell lines. The *D. melanogaster* host has thus provided the core information that can be used to study responses to natural microbial and metazoan pathogens as they become identified, as well as to test ideas of selection and evolutionary change. These analyses are of general importance to understanding mechanisms of other insect host–pathogen interactions and determinants of variation in host resistance.

Keywords

Innate; immunity; *Drosophila*; genomes; NF- κ B; signal transduction; JAK-STAT; antimicrobial peptides

Introduction

Metazoan animals have mechanisms that allow them to defend themselves against the main threats to their integrity as individuals. These common threats (infection, parasitism, and neoplasia) disturb the delicate balance among cells, tissues and organs. A large variety of defense responses have evolved that differ in their speed and specificity in recognizing and processing a given threat. To simplify this variety of defense responses, immunologists have classified them as either cellular or humoral and either possessing or lacking memory.

Elements of innate immunity

Humoral or cellular responses to infectious agents can either be innate or adaptive. The terms “innate”, “natural” or “nonspecific” immunity refer to the variety of physical, cellular or molecular features which provide the first lines of defense against infection or injury. In vertebrates, innate immune responses support and operate alongside adaptive immune responses, to generate antigen-specific mechanisms that eventually lead to the destruction and elimination of the pathogen.

Innate immunity is present in all life forms and the information for innate immune responses is inherited. In mammals, the skin and the epithelial lining of the mucosal tissues act as the

primary nonspecific barriers to infection. If infectious agents gain entry into the body through damaged skin, internal innate immune responses are activated. Internal innate immune agents and responses include low pH, proteolytic enzymes and the bile, the secretion of antibacterial substances and acute phase proteins, activation of complement and phagocytosis.

Phagocytosis is a fundamental innate immune mechanism used by a variety of cell types, including macrophages and dendritic cells. Widely distributed in the human body and associated with different tissues, these cells consume micro-organisms, other foreign substances and apoptotic cells. Dendritic cells reside in an immature state in most tissues and develop from the same hematopoietic precursors that give rise to the macrophages. When they come in direct contact with the pathogens, dendritic cells mature, increase their capacity to present antigen and gain the ability to activate naïve T cells of the adaptive immune system (Janeway *et al.*, 2004).

Cells in the mammalian innate immune system detect “microbial nonself” by recognizing pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 2002). PAMPs are products of microbial metabolism conserved over evolution and distributed in a wide variety of pathogens. Lipopolysaccharide (LPS), peptidoglycan, flagellin, double-stranded RNA and hypomethylated DNA islands are examples of PAMPs. PAMP receptors, called pattern recognition receptors (PRRs) are present on surfaces of host cells and recognize specific or related PAMPs. Toll-like receptors (TLRs) are one class of PRRs expressed on cells of the innate immune system. TLRs directly sense non-self, and when activated, PRRs induce intracellular signaling, that results in the activation of genes involved in host defense. Genes typically activated include those encoding antimicrobial peptides, inflammatory cytokines or chemokines (Fig. 1).

Adaptive or acquired immunity is characterized by specificity and memory. Developed more recently with the evolution of the gnathostomata, the acquired immune response has nearly infinite adaptability. Yet, adaptive immunity depends on the innate immune responses, as in organisms where both systems are present, adaptive immunity is fashioned by innate sensing mechanisms and the ensuing responses. For a fully functional immune system, these components must act in synergy (Hoebe *et al.*, 2004; Fig. 1).

Innate immunity in *Drosophila*

Because of the absence of adaptive immune response (Fig. 1), *Drosophila* serves as a powerful model for studying aspects of the innate immune system that might otherwise be masked by the adaptive immune response. This system has been a focus of intense analysis: Genome-wide studies of immune responses are now routinely performed in *Drosophila*: microarrays and proteomic approaches complement RNA interference and conventional genetic methods, expanding the range of experimental possibilities.

Like vertebrates, *Drosophila* spp. defend themselves against a range of parasites and microbes by invoking a variety of innate immune responses (Fig. 2), some of which are clearly shared with vertebrates. The immune responses include: (i) epithelial barriers (cuticle, trachea, gut) and clotting; (ii) humoral reactions of antimicrobial peptides produced by the fat body; and (iii) cellular reactions (blood cell- or hemocyte-dependent phagocytosis of microbes and encapsulation of larger pathogens). In addition, melanization accompanies coagulation, wound healing and encapsulation, adding to the armor of host defense (Hultmark, 2003; Hoffmann, 2003; Brennan & Anderson, 2004; Imler & Bulet, 2005).

Perhaps the most powerful and the best-studied of these reactions is the systemic immune response, in which antimicrobial peptides are secreted from cells of the fat body into the hemolymph. This response is induced by the invading microbial pathogens. There is ample

evidence that in the adult fly, antimicrobial peptides limit bacterial or fungal growth. In general, flies with mutations in genes encoding specific components of the NF- κ B (Toll and Imd) pathways are impaired in their ability to fight bacterial or fungal infections. Microarray analyses of all fly genes confirm the central importance of these post-transcriptional mechanisms in the expression of a vast majority of immunity genes. A brief overview of these physical and molecular aspects of innate immune responses in *Drosophila* follows.

Epithelial barriers and coagulation

Epithelia deter bacteria and act as the first line of defense. Epithelial cells of the epidermis and those that line the digestive (gut), respiratory (trachea), and reproductive systems are normally in contact with microbes and they serve as natural routes of microbial infection (Tzou *et al.*, 2000; Onfelt *et al.*, 2001). Transgenic strains carrying AMP promoter-GFP fusions reveal that AMP genes are expressed in surface epithelia locally in a cell- or tissue-specific manner. This expression may be constitutive (e.g., Drosomycin in the female spermatheca; Tzou *et al.*, 2000) or inducible (e.g., Diptericin or Drosomycin in tracheae and gut). The production of the inducible epithelial AMPs is at least partially under the control of the Imd pathway (reviewed in Lemaitre & Hoffmann, 2007).

Pathogenic bacteria ingested in food do not appear to be harmful to the wild-type fly host, apparently because of an antioxidant system involving the activities of dual oxidase (Duox) and an extracellular immune-regulated catalase (Ryu *et al.*, 2006; Haet *et al.*, 2005a, 2005b). RNA interference-mediated silencing of either of these genes disturbs the homeostatic redox balance in the gut lumen and increases susceptibility of flies to bacteria. These results indicate that immune-regulated catalase and Duox activities are important for regulating reactive oxygen species levels and protecting fly hosts against gut infection (Ha *et al.*, 2005a, 2005b).

After the primary epithelial barrier is breached, the infected animal erects a second barrier in the form of a clot that traps microbes and also limits the loss of hemolymph (Scherfer *et al.*, 2004). Proteomic studies have identified a dozen proteins making up the clot fibers. Chief among these is hemolymph, a multidomain protein expressed in plasmatocytes, one of the larval hemocytes. Fondue, another abundant hemolymph protein produced by plasmatocytes is also required for optimal coagulation. Animals deficient in either of these proteins remain healthy even after immune challenge; however in both cases, RNAi mutants form larger melanized scabs after wounding with a tungsten needle relative to controls (Goto *et al.*, 2003; Scherfer *et al.*, 2004, 2006), suggesting that these proteins play a role in wound healing. Clot formation can proceed independently of melanization, as pro-phenol oxidase-compromised mutants form normal clots (Scherfer *et al.*, 2004). The process of hemolymph coagulation is thought to be similar to the production of extracellular fibrous traps by mammalian neutrophils after cytokine activation. These traps kill extracellular bacteria independently of phagocytosis (Brinkmann *et al.*, 2004).

The systemic immune response against bacteria and fungi

A number of microbial pathogens (viral, bacterial and fungal) and metazoan pathogens (parasitoid wasps) infect and kill *D. melanogaster* (Table 1). They differ in their modes of infection and effects on host physiology. A healthy immune-competent fly host coordinates a set of immune defenses; however natural pathogens have in turn evolved to circumvent some of these host defense mechanisms.

Bacteria

Bacteria can infect flies during ingestion or via injuries. Bacterial strains of *Serratia marcescens* (Db11), *Erwinia carotovora* (Ecc15) and *Pseudomonas entomophila* trigger an

immune response in *Drosophila* after oral infection (Basset *et al.*, 2000, 2003). However, most studies in the adult fly have been done using injection as the mode of infection. Bacterial pathogens studied after injection include *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Salmonella typhimurium* and *Mycobacterium marinum* (reviewed in Lemaitre & Hoffmann, 2007). Even though a manual injection perturbs the balance of host immune (and other) responses, each bacterial species evokes a fairly characteristic set of responses, making the fly host ideal for genetic dissection of processes and pathways governing innate immunity.

Wolbachia is a natural bacterial endosymbiont of *Drosophila*, frequently found in flies in natural and laboratory conditions, and normally transmitted vertically from a female host to her offspring. *Wolbachia* are able to colonize different tissues although they prefer to infect cells of reproductive tissues. Recent studies (Lazzaro *et al.*, 2006; Cox & Gilmore, 2007) mark the beginning of the characterization of other natural microbial flora in the *D. melanogaster* gut and hemolymph. Both Gram-positive (*Enterococcus* spp., *Lactococcus lactis*) and Gram-negative (*Acetobacter pasteurianus*, *Serratia marcescens*, *Providencia burhodogranaria*) bacteria colonize *Drosophila* (Table 1). It is not known whether the fly host resists colonization of these species. Determining this is essential to whether flies have a stable microbial flora and if these populations play an ecological role in the bacteria–fruit fly consortia.

Fungi

Beauveria bassiana, a fungus that parasitizes insects, can grow on adult flies if its spores are applied to the host. Because of this natural ability to infect flies, *B. bassiana* has been used as the “model” fungus for studying fungal-specific responses in *Drosophila* (Lemaitre *et al.*, 1997; De Gregorio *et al.*, 2001, 2002). *Candida albicans*, a common human pathogen, has also been used to examine phagocytosis in intact *Drosophila* and in cell culture experiments (Alarco *et al.*, 2004; Stroschein-Stevenson *et al.*, 2006).

The core circuitry—intracellular components—DNA-binding studies of promoters of antimicrobial peptide genes (Engstrom *et al.*, 1993; Kappler *et al.*, 1993) and the discovery of Dif (Ip *et al.*, 1993) and Relish (Dushay *et al.*, 1996) gave impetus to the idea that NF- κ B signaling may be important in immune-inducibility of AMPs. Genetic studies of mutant animals demonstrated that flies can discriminate between classes of surface molecules on pathogens, and further, two distinct NF- κ B pathways, the Toll and IMD pathways, control AMP production by recruiting the functions of NF- κ B transcription factors Dorsal/Dif and Relish respectively (Fig. 3).

The two pathway model hinged on the discovery of the *Imd* (for immune deficiency) mutation (Lemaitre *et al.*, 1995). A combination of forward genetic screens and reverse genetic analyses led to the identification of eight additional *Imd* pathway components (Fig. 3). The Toll pathway is responsible for resistance to fungal and Gram-positive bacterial infections. The two pathway model is strengthened by evidence from additional studies including genome-wide microarray analyses (De Gregorio *et al.*, 2001, 2002). The cytoplasmic components of both these signaling mechanisms have homologs in other insects (mosquitoes, Christophodos *et al.*, 2002; bees, Evans *et al.*, 2006) where they are likely to play a similar function in host defense. The mammalian homologs of these proteins also regulate innate immune responses. Evolution has thus preserved the essential circuitry of innate immunity.

The Toll pathway—The intracellular components of the Toll pathway were identified in genetic screens for maternal-effect mutations affecting dorsal-ventral polarity of the fly embryo (Govind, 1999). Unlike vertebrate Toll-like receptors, the fly trans-membrane receptor Toll1 does not bind directly to pathogens. Instead, Toll1 is activated by a processed form of Spätzle,

a cysteine-knot protein with structural similarities to nerve growth factor. Toll activation leads to destabilization of the cytoplasmic complex of I κ B protein Cactus and NF- κ B proteins Dorsal/Dif via intracellular components MyD88, Tube and the Pelle kinase. Activation of the Toll pathway results in degradation of the I κ B-family protein Cactus and localization of Dorsal and Dif to the nucleus. Dorsal/Dif activate *Drosomycin* expression (Fig. 3). Dorsal and Dif function redundantly and cell-autonomously in the larval fat body (Manfrulli *et al.*, 1999). Dif is essential in the adult response (Rutschmann *et al.*, 2000). Toll signaling remains repressed by functions of Cactus and the SUMO-conjugating enzyme dUbc9 (Chiu *et al.*, 2005). The extracellular components of the Toll pathway necessary for Spätzle activation in embryonic development and immunity are different (see below). Mutations in most of these well-conserved intracellular signaling components render flies susceptible to fungal and Gram-positive bacterial infections (Hoffmann, 2003; Hultmark, 2003; Tanji & Ip, 2005).

The IMD pathway—Unlike the Toll pathway that also regulates other processes in the fly life cycle, the Imd pathway appears to be dedicated to defending hosts against Gram-negative bacteria (Martinelli & Reichhart, 2005; Lemaitre & Hoffmann, 2007). Imd protein contains a death domain similar to the one in Receptor Interacting Protein of the TNF-receptor. The main receptor of the Imd pathway is peptidoglycan recognition protein-LC (PGRP-LC; see below). Components downstream of Imd are TAK1, TAB2, DIAP2, IKK β /ird5, IKK γ /Kenny, the FADD adaptor, the Dredd caspase, and the transcription factor Relish (Fig. 3). The IKK β /ird5 and IKK γ /Kenny form a complex equivalent to the mammalian IKK signalosome. The IKK complex is responsible for phosphorylation of Relish. Relish is a compound Rel-family protein with a Rel domain and ankyrin-repeat containing inhibitory domain. Phosphorylated Relish is cleaved by Dredd. Relish cleavage in the cytoplasm releases the Rel domain from its inhibitory domain, which remains in the cytoplasm. The Imd pathway remains repressed by the ubiquitin-proteasome system (Khush *et al.*, 2002). The order of action of these proteins (see Fig. 3) was deduced from epistatic experiments in which typically one “gain-of-function” component was combined with the “loss-of-function” mutation in a second component. If the outcome of such a combination results in the suppression of the “gain-of-function” phenotype (i.e., constitutive expression of the AMP gene is reduced), then the second component is assumed to be acting “downstream” of the first.

In general, the kinetics of *Drosomycin* and *Diptericin* mRNA expression, the canonical “read-outs” of the Toll and Imd pathways, respectively, are quite different: For example, *Drosomycin* expression peaks a day after injury, whereas *Diptericin* expression peaks within the first 12 hours of septic injury. However, together, both these NF- κ B pathways account for about 80% of the genes induced by septic injury (De Gregorio *et al.*, 2001, 2002). While there is no evidence of cross-talk between the pathways, some AMP genes can be induced by both of them, whereas others show specific input from a single pathway.

Other pathways—In addition to the Toll and Imd pathways, other signaling mechanisms complement and contribute to the core circuitry. The expression of a subset of the immune-responsive genes such as the thiol-ester protein *Tep1* and the stress response gene *TotA* also require input from JAK-STAT signaling (Lagueux *et al.*, 2000; Agaisse *et al.*, 2003). While the initial steps leading to receptor activation are less clear, events downstream of Domeless are carried out by the Janus Kinase Hopscotch and the transcription factor STAT92E. Thus, some aspects of the systemic immune response are complex and require the coordination and integration of different cell types and regulatory mechanisms (Agaisse *et al.*, 2003). JAK-STAT-deficient flies are resistant to bacterial and fungal infections but succumb to viral infections (Dostert *et al.*, 2005). Like the Toll pathway, the JAK-STAT pathway also regulates other physiological and developmental functions in the fly.

PAMPs, PRRs and extracellular processes—One of the long-standing questions in the field of insect immunity has been how, in the absence of antibodies and the major histocompatibility complex, the insect immune system distinguishes between foreign and self-antigens. In flies, members of the PRR families (PGRPs and GNBP) sense the presence of non-self. PGRPs can bind to a specific form of peptidoglycan (PGN, a PAMP molecule) that is present in both Gram-positive and Gram-negative bacteria. There are 13 PGRPs in the fly, and all share a 160-amino acid PGRP domain (Werner *et al.*, 2000; Royet *et al.*, 2005). PGRPs can bind (and recognize) PGN. They may also possess key zinc-binding residues that confer a zinc-dependent N-acetylmuramoyl-L-alanine amidase activity. It has been proposed that the amidase activity of this latter class of catalytic PGRPs (PGRP-SC1A/B, LB, SB1/2 and SC2) removes peptides from the glucan chains of PGN, altering its biological activity (Mellroth *et al.*, 2003; Mellroth & Steiner, 2006; Zaidman-Remy *et al.*, 2006). Members of the non-catalytic class of PGRPs serve as receptors and include PGRP-LC (the major receptor of the Imd pathway) as well as PGRP-SA, SD, LA, LD, LE and LF. The three-dimensional structures of four of these proteins have been solved. These structural studies provide insights into the binding/recognition-specificity versus catalytic functions of PGRP family members (reviewed in Lemaitre & Hoffmann, 2007).

Gram-positive and Gram-negative PGN show subtle but important differences in chemical structure and localization. The Gram-positive PGN (lys-type) is multilayered and exposed at the bacterial surface, whereas Gram-negative PGN (DAP-type) is single-layered and found within the periplasmic space under the outer bacterial membrane. Both these differences are thought to be important in the specificity of pathogen recognition. The Toll pathway is activated by the lys-type PGN, whereas the Imd pathway is activated by DAP-type PGN (Leulier *et al.*, 2003; Kaneko *et al.*, 2004; Stenbak, 2004). The second important difference in their activation is that the Toll pathway is activated by secreted members of the PGRP family (PGRP-SA and SD), whereas the Imd pathway is activated by membrane-bound or intracellular receptors (PGRP-LC and LE).

Extracellular events—It is commonly accepted that Spätzle activation and binding triggers the Toll pathway (Weber *et al.*, 2007). The steps intervening pathogen recognition and Spätzle processing are not completely understood. However, it is believed that a zymogen activation cascade like the one that activates Spätzle in the embryo (Govind, 1999; Hashimoto *et al.*, 2003; Weber *et al.*, 2003) is also responsible for processing Spätzle after immune challenge. This idea is supported by the discovery of the Spätzle Processing Enzyme (SPE) that is highly similar to Easter, the equivalent protease in the early embryo (Jang *et al.*, 2006; Kambris *et al.*, 2006).

Events upstream of SPE appear to be distinct, depending on the nature of the pathogen: Gram-positive bacteria are recognized by PGRP-SA, PGRP-SD and Gram-negative binding protein-1 (GNBP-1, an apparent misnomer). Immune-deficient phenotypes of PGRP-SA and GNBP-1 mutant flies are similar and there is evidence that these two proteins act in concert (Gobert *et al.*, 2003; Pili-Floury *et al.*, 2004). The presence of fungi is recognized by GNBP3 (Gottar *et al.*, 2006), a circulating PRR. GNBP3 mutants are immune-compromised and cannot activate Toll signaling. GNBP3 belongs to a small family with proteins containing an N-terminal portion that binds β (1,3)-glucan and the C-terminal catalytic β -glucanase domain. Fungal infection is mediated by an extracellular cascade involving the Clip-serine protease Persephone (Ligoxygakis *et al.*, 2002) and the serine protease inhibitor Necrotic (Levashina *et al.*, 1999). Both these enzymes control Spätzle activation although in opposite ways: Persephone function is needed for Spätzle activation, whereas Necrotic is a negative regulator and its inhibitory function is necessary to limit Toll signaling.

The Imd pathway is activated primarily by Gram-negative PGN whose presence is detected by the membrane-bound PGRP-LC. The PGRP-LC gene is alternatively spliced to result in three isoforms: LCa, LCx and LCy. These isoforms share the intracellular domain but differ in their extracellular structures (Werner *et al.*, 2000; Kaneko *et al.*, 2004). The isoforms show differences in specificity to monomeric versus polymeric PGN and two PGRP-LC molecules may come together to bind polymeric PGN and activate signaling (Reiser *et al.*, 2004; Mellroth *et al.*, 2005; Chang *et al.*, 2005, 2006). PGRP-LE detects DAP-type PGN and can function as a receptor both inside and outside the cell (Kaneko *et al.*, 2006). The Imd pathway is thought to be attenuated by the amidase activities of PGRP-LB (Zaidman-Remy *et al.*, 2006) and PGRP-SC (Bischoff *et al.*, 2006). The amidase activity processes peptides from the glycan chain of PGN. This mechanism is thought to contain the intensity and duration of the Imd response.

Antimicrobial defenses against bacteria and fungi

Humoral defense responses in insects include localized melanization, lectin binding, and induction of antimicrobial peptides (AMPs) via activation of the IMD and Toll pathways (Fig. 3). Humoral factors such as antimicrobial proteins (AMPs) and complement-like proteins called *Drosophila* thiol-ester-containing proteins, (or dTEPs) are thought to be the primary immune effector molecules. Roughly 20 AMP genes in *Drosophila* represent seven classes: antifungal AMPs (drosomycin, metchnikowin), anti-Gram-negative AMPs (attacins, cecropins, dipterocins, drosocin), and an anti-Gram-positive AMP (Defensin) (Martinelli & Reichhart, 2005). AMPs are synthesized mostly in fat body cells and released into the hemolymph. However, cells of barrier tissues such as the midgut (Diptericin, Attacin), the tracheae (Drosocin, Drosomycin), the oviduct (Drosocin, Cecropin), the seminal receptacle and the spermatheca (Cecropin, Defensin, Drosomycin) also express AMPs (Tzou *et al.*, 2000) as part of the local defense mechanism. Antimicrobial peptides are small and cationic and are thought to affect microbial membrane properties (Imler & Bulet, 2005). The three-dimensional structures of Cecropin, Defensin, Drosocin, Drosomycin have been determined, although the structural basis of their activities is not understood.

The *Drosophila* genome encodes six members of the dTEP gene family (Lagueux *et al.*, 2000). dTEPs are acute-phase glycoproteins that belong to the C3/ α 2-macroglobulin superfamily. Through their thioester motifs, TEPs form covalent links to bacterial surfaces. In mammals, similar modification of bacterial surfaces by the complement protein C3 marks microbes for opsonization. Because these molecules are expressed in the fat body and presumably secreted into the hemolymph, dTEPs are considered to be part of the humoral response. The expression of TEPs is under the control of the JAK-STAT pathway (Lagueux *et al.*, 2000).

Immune responses against viruses

More than 25 viruses have been documented to infect *Drosophila* (Brun & Plus, 1980; Ashburner *et al.*, 2005), and the effects of few of these have been studied recently (Lemaitre & Hoffmann, 2007; Cherry & Silverman, 2006). Viruses can spread by vertical infection from the egg to adult as is the case with the Sigma virus. Alternatively, they are transmitted horizontally (feeding or contact) as in the case of *Drosophila* C virus (DCV) or *Drosophila* X virus (DXV). DCV is a picorna-like RNA virus (Johnson & Christian, 1998). The DCV genome is single-stranded positive genomic RNA. DXV is a Birnavirus, with two linear, double-stranded RNA molecules (Zambon *et al.*, 2005). In general, even though virus-infected flies may appear otherwise normal, injections of adults with virus make them sensitive to carbon dioxide exposure and cause early death of the animal (Seecoff, 1965; Cherry & Perrimon, 2004; Zambon *et al.*, 2005).

The Toll and JAK-STAT pathways are involved in resistance against DXV (Zambon *et al.*, 2005) and DCV (Dostert *et al.*, 2005). However, neither of these mechanisms appears to be central to the antiviral defense in the fly. Instead, flies employ the RNA interference pathway (Fig. 4) to resist virus infection. (Wang *et al.*, 2006; Galiana *et al.*, 2006; Zambon *et al.*, 2006). Several core members of the RNAi machinery such as Dicer-2, r2d2, Argonaute-2 and piwi are important in executing the antiviral response to DXV, DCV and other unrelated infectious viruses (cricket paralysis virus, flock house virus): mutations in these loci lead to increased sensitivity to viral infection and enhanced mortality with higher viral load in mutant adults. Thus, like the role of NF- κ B signaling in innate immunity, RNAi shows structural and functional conservation in host defense from plants to mammals.

Immune suppression by DCV

Recent studies on the *Drosophila* C virus provide interesting insights into *Drosophila*-DCV interaction. DCV injections into the fly abdomen result in dose-dependent lethality (Cherry & Perrimon, 2004). As mentioned above, the RNA-induced silencing complex RISC component Argonaute 2, which has endonuclease activity is essential for protecting the host from virus-induced mortality (Galiana-Arnoux *et al.*, 2006; Wang *et al.*, 2006; van Rij *et al.*, 2006). In *ago-2* mutant flies, an increased accumulation of viral RNA levels is observed and this increase is accompanied by higher RNA titers. Interestingly, DCV encodes a suppressor of the RNAi machinery (van Rij *et al.*, 2006). This suppressor specifically inhibits only RNAi silencing (that is initiated by long dsRNA) but apparently not miRNA biogenesis or function. Tentatively named DCV-1A, the suppressor gene encodes an RNA-binding protein with a canonical dsRNA-binding domain (dsRBD). This protein (and its N-terminal 99 amino acids containing the dsRBD) can suppress RNAi. Suppression of RNAi is dependent on the highly conserved residues of the RNA-binding domain. Transgenic flies expressing DCV-1A can suppress the dsRNA-induced silencing of the *white* gene in adult flies. Finally, DCV-1A has dsRNA-binding activity and it can inhibit Dicer processing of dsRNA. The authors (van Rij *et al.*, 2006) propose that by binding to the long double-stranded RNA, DCV-1A inhibits Dicer-2-mediated dsRNA processing into siRNAs. Thus DCV-1A protein allows DCV to overcome the RNAi defense mechanism of the host.

Phagocytosis of microbes

Microbes in the hemolymph are consumed by phagocytic cells called plasmatocytes. Plasmatocytes are the predominant hemocyte type in healthy animals and they carry receptors for recognizing bacteria, yeast, viruses and apoptotic cells. Pathogen recognition is followed by internalization. Recent studies have identified a number of phagocytic receptors. In addition, cultured cells of *Drosophila* called S2 are highly phagocytic. This makes it easy to perform genome-wide RNAi screens for genes involved in the phagocytic process. These studies show that there are many similarities in pathogen recognition and engulfment between flies and mammals.

Receptors

(i) The IgSF-domain containing protein isoforms Dscam (*E. coli*, Watson *et al.*, 2005); (ii) members of the class C scavenger receptor (dsR-CI, dsRNA; Ramet *et al.*, 2001); (iii) PGRP family (PGRP-SC1a; *Staphylococcus aureus*; Garver *et al.*, 2006); (iv) epidermal growth factor-domain containing protein Eater (dsRNA, *E. coli*, *S. aureus*; Kocks *et al.*, 2005); and (v) CD36-related protein Peste (*Mycobacterium*, *Listeria*; Philips *et al.*, 2005). These receptors show selectivity in pathogen binding/internalization (reviewed in Cherry & Silverman, 2006; Lemaitre & Hoffmann, 2007).

In addition to these receptor molecules, TEPVI (also called Mcr, for macroglobulin complement-related and a TEP family member without the active thioester motif) is important in the uptake of the fungus *Candida albicans*, but not that of *S. cerevisiae*. Other TEP family members also modestly contribute to phagocytosis of *E. coli* (TepII) or *S. aureus* (TepIII), suggesting that these complement-like proteins facilitate microbe recognition or uptake (Stroschein-Stevenson *et al.*, 2006). Significantly, *Anopheles* mosquito has 19 TEPs, and TEP1 of *A. gambiae* has been shown to mediate parasite killing (Blandin *et al.*, 2004).

Internalization

Cell culture studies (Cherry & Perrimon, 2004; Agaisse *et al.*, 2005; Philips *et al.*, 2005; Benghezal *et al.*, 2006; Stroschein-Stevenson *et al.*, 2006) have identified proteins required after pathogen internalization. These include proteins important for actin cytoskeletal reorganization (such as actin capping proteins, Arp2/3 complex, cdc42, and cofilin) and those involved in clathrin-mediated endocytosis pathway or in vesicle trafficking. *In vivo*, the requirement of WASp homolog D-Scar and Chickadee has been shown (Pearson *et al.*, 2003). These recent findings set the stage for a fine-grained analysis of steps involved in pathogen recognition, internalization, and the subsequent intracellular steps that lead to microbe destruction.

Immune responses against parasitoids

Parasitoids

In addition to microbial pathogens, parasitoid wasps infect *Drosophila* larvae and pupae. In some natural populations, more than half of *Drosophila* larvae collected are infected, indicating that wasps can be a potent cause of mortality. Parasitoids develop within the larval body cavity. Most parasitoid species studied in the lab are highly virulent on the *D. melanogaster*. With their sharp ovipositors, female wasps inject one or more eggs into the host hemocoel. *Leptopilina*, *Asobara*, *Ganaspis* and *Trichopria* species infect *Drosophila* (Table 1), although, of these, host interactions with the *Leptopilina* spp. are the best studied. *L. boulardi*, *L. heterotoma* and *L. victoriae* are highly virulent but. *L. boulardi* differs from the other two in its host range restricted to the melanogaster group of flies (Schlenke *et al.*, 2007).

Cellular responses—encapsulation

Being much larger than the 10- μ plasmatocyte, the wasp egg cannot be phagocytosed. Instead, hemocytes (plasmatocytes, crystal cells and lamellocytes) cooperate and physically surround and encase the egg. This encapsulation reaction involves hemocyte division and differentiation. This reaction is relatively slow and capsules are not observed until 1–2 days after infection. Whether (and how quickly) the egg becomes encapsulated also depends on where in the larval hemocoel the egg is localized after oviposition, the physicochemical features of the egg itself and the presence of immune-suppressive factors or virus-like particles.

Encapsulation of the wasp egg involves at least three phases: (i) recognition of wasp egg; (ii) the differentiation of highly flattened and adhesive blood cells called lamellocytes; and (iii) construction of the capsule. Lamellocyte precursors reside in the larval lymph gland (Lanot *et al.*, 2001; Sorrentino *et al.*, 2002) although their existence in circulation has not been ruled out. Components of the Toll pathway are also expressed in the lymph gland (Qiu *et al.*, 1998) and Toll signaling is required for wasp egg encapsulation. Many other genes and pathways, especially JAK-STAT signaling also contribute to differentiation of hemocytes after immune challenge (Crozatier & Meister, 2007). Mutations in components of Toll and JAK-STAT pathways affect hemocyte counts and compromise the host's ability to mount an effective encapsulation reaction in response to *L. boulardi* infection (Sorrentino *et al.*, 2004).

Construction and melanization of a capsule involve hundreds of cells and require the coordinated action of many genes. The capsule is constructed largely of lamellocytes and these highly mobile cells organize to construct a multi-layered capsule with septate junctions and integrin-dependent cell interactions (Irving *et al.*, 2005). Melanization is the blackening of the capsule surrounding the parasite egg. Melanin formation involves multiple steps that include the oxidation of monophenols and diphenols. These steps are catalyzed by the enzyme pro-phenol oxidase or proPO. The importance of the phenoloxidase cascade in the encapsulation reaction is controversial; some evidence against its involvement comes from the analysis of phenoloxidase-deficient mutants which encapsulate parasite eggs but fail to melanize the capsule (Rizki *et al.*, 1980). However, in a genome-wide microarray study, several genes encoding enzymes of the melanization cascade were found to be upregulated by *L. boulandi* infection (Schlenke *et al.*, 2007 see below).

Systemic responses

Surprisingly, larvae infected by *Asobara tabida* or *Leptopilina boulandi*, but not *L. heterotoma* result in the upregulation of the Toll/Imd pathways and expression of antimicrobial peptides (Wertheim *et al.*, 2005; Schlenke *et al.*, 2007). Whole genome microarrays of hosts infected by each of these wasps reveals that hosts infected by *A. tabida* or *L. boulandi* induce numerous genes encoding proteolytic enzymes, components of the Toll and JAK/STAT pathways, and the melanization cascade as part of a combined cellular and humoral immune response. However, flies attacked by *L. heterotoma* do not initiate much of an immune transcriptional response. The difference could be attributable to the rapid VLP-mediated lysis of host hemocytes by *L. heterotoma* (see below). These studies suggest that the systemic immune responses initiated by *L. boulandi* or *A. tabida* play a role in host defense against parasitoid infections. The molecules involved in parasitoid egg recognition are not known, although microarray studies reveal that parasitoid infection activates transcription of specific PGRP genes, suggesting that their products may serve to recognize the presence of the foreign invaders (Wertheim *et al.*, 2005; Schlenke *et al.*, 2007).

Immune suppression by *L. heterotoma*/*L. victorae*

The cynipid sister species *L. heterotoma* and *L. victorae* produce virus-like particles (VLPs) that are implicated in suppression of egg encapsulation. VLPs are produced in the long gland-reservoir complex, a tripartite structure connected to the ovipositor and associated with the ovary (Rizki & Rizki, 1990; Morales *et al.*, 2005). VLPs are deposited in the host hemocoel during oviposition. VLPs of *L. heterotoma* and *L. victorae* have the ability to lyse lamellocytes *in vitro* and *in vivo* (Rizki & Rizki, 1990; Morales *et al.*, 2005). Infection by these parasitoids also results in depletion of the hematopoietic precursors in the lymph gland (Chiu *et al.*, 2002). *L. heterotoma* infection does not activate AMP gene expression (Schlenke *et al.*, 2007) but it is not known whether the active immune suppressive factors that target hemocytes also suppress or inactivate gene expression in the fat body. Future research in this system will reveal how the activation of cellular and humoral immune pathways are coordinated by some wasps (*A. tabida* and *L. boulandi*) and how immune suppressive or evasive mechanisms of other species (*L. heterotoma* and *L. victorae*) contribute to parasitoid virulence and determine host range.

Future perspectives

Many human conditions such as allergy, autoimmune disorders, rheumatoid arthritis-related inflammation and septic shock are linked to dysfunctional innate or innate/adaptive immune responses. For many of these conditions, the molecular genetic lesions have not been pinpointed. For example, it is not known if these problems arise due to a failure of self/non-self discrimination, if the downstream signaling mechanisms are anomalous, or if the

connections at the innate/adaptive interface are aberrant. The crucial role of the innate immune system in limiting parasitic or microbial diseases in humans is also not well understood and its analysis is complicated by the presence of adaptive mechanisms that are closely tied to innate mechanisms. The *Drosophila* immune system shares many features of the mammalian innate immune system at tissue, cell and molecular levels, is multifaceted and is now relatively well characterized. It is therefore a powerful model for study of the more subtle and complex aspects of the infection process, including disease progression after infection by (one or more) natural pathogens, coordination and intersection of different “arms” of the immune system, and many aspects of host physiology and immune-competence. Given that the basic scaffold of innate immune regulation is highly conserved, it will be interesting to uncover the nature and evolution of virulence in diverse organisms. These studies will provide a clearer picture of how complex biological processes and systems, such as adaptive immunity, were assembled, maintained, and altered in evolution.

Acknowledgments

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Fig. 1.

The relationship between innate and adaptive immunity. The immune response is initiated by recognition of the pathogen. The recognition step depends on specific receptors and other molecular components. In flies where adaptive immune response is absent, hosts rely completely on innate immunity. In vertebrates, adaptive immune response is dependent on the successful outcome of the innate system. Activation of Toll-like receptors by PAMPs induces the functions of costimulatory molecules that enhance adaptive response. Costimulatory molecules may be membrane-bound or secreted from cells of the innate immune system. They are required to fully activate signal transduction events within T or B cells of the adaptive immune system. The induction of costimulators along with presentation of antigenic peptides on antigen-presenting cells (e.g., dendritic cells) links the initial recognition of pathogens with the activation of the adaptive immune system.

Fig. 2.

The immune system of *Drosophila* has many facets, each tailored to defend against pathogens infecting the host at different life cycle stages. For details, see text.

Fig. 3.

The two pathway model. (i) Infection by fungi or Gram-positive bacteria activates the SPE-Spatzle-Toll-Tube-Pelle-Cactus-Dorsal/Dif cascade in the larval or adult fat body. Dorsal and Dif activate the expression of antifungal genes drosomycin and metchnikowin. (ii) The IMD-Relish pathway is activated by Gram-negative bacteria and regulates expression of dipterocin, attacin, drosocin, cecropins, and defensin. Activation of these pathways results in nuclear localization of NF- κ B-family proteins Dorsal, Dif, or Relish. These pathways are differentially activated by pathogen-specific molecules that bind to PRRs. Note that each is inhibited by negative regulators that are also unique.

Fig. 4.

Defense against viruses (or viral products) requires Toll (DXV, *Drosophila X* virus) or JAK-STAT (DCV, *Drosophila C* virus or flock house virus FHV) signaling. Viruses gain entry into host cells utilizing cellular factors. Uncoating and replication of the viral RNA genome leads to formation of dsRNA. The host RNAi activity targets viral dsRNA for destruction. However, a DCV factor (double-stranded RNA-binding protein encoded by the DCV-1A gene) inhibits the antiviral RNAi activity. See details for text.

Table 1Some pathogens and commensals of *Drosophila*.

Class	Bacterial	Fungal	Viral	Parasitoid
Natural	<i>Erwinia carotovora</i>	<i>Beauveria bassiana</i>	DCV, DXV	<i>Leptopilina</i>
	<i>Enterococcus</i> spp.	<i>Metarhizium anisopliae</i>	Sigma	<i>Ganaspis</i>
	<i>Lactococcus lactis</i>			<i>Asobara</i>
	<i>Acetobacter pasteurianus</i>			<i>Trichopria</i>
	<i>Serratia marcescens</i>			
	<i>Providencia burhodogranaria</i>			
Other	<i>Escherichia coli</i>	<i>Candida albicans</i>	Cricket paralysis virus	
	<i>Micrococcus luteus</i>	<i>Aspergillus fumigatus</i>	Flock house virus	
	<i>Pseudomonas aeruginosa</i>			
	<i>Serratia marcescens</i>			
	<i>Mycobacterium marinum</i>			
	<i>Salmonella typhimurium</i>			
	<i>Enterobacter</i>			

DCV, *Drosophila* C virus; DXV, *Drosophila* X virus.